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Relationship between Calcium in Atherosclerotic Plaques and Bone Mineral Density

– A Clinical and Histological Perspective –

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I was gratified to be able to answer promptly, and I did. I said I didn't know.

— Mark Twain

RESUMO

A relação entre osteoporose e aterosclerose é ainda pouco clara. Contudo, além da associação epidemiológica e de factores de risco, nomeadamente, a influência da idade em ambas as patologias, existem outros dados de cariz clínico, genético e patogénico que sugerem a possibilidade de uma via comum de desenvolvimento. A calcificação vascular tem sido alvo de estudo, existindo várias investigações que demonstram um aumento de depósitos de cálcio em placas de ateroma nos doentes com osteoporose.

O presente estudo consistiu na avaliação histológica dos depósitos de cálcio nas placas de ateroma de doentes com e sem osteoporose, por meio de uma análise semi-quantitativa dos depósitos de cálcio.

Neste estudo foram incluídos 32 doentes submetidos a endarterectomia carotídea dos quais se obtiveram amostras de sangue e placa de ateroma. Os doentes realizaram também uma densitometria óssea e foi aplicado um protocolo clínico estruturado.

As amostras foram coradas com *Alizarin Red S* e as lâminas fotografadas com o *NanoZoomer*®, por forma a conseguimos observar nas placas de ateroma os depósitos de cálcio. A partir da análise semiquantitativa realizada pelo *Image J*® constatámos que os doentes com osteoporose apresentam áreas relativas de depositos de cálcio numericamente superiores (mediana: 45.46%) aos indivíduos sem osteoporose (mediana: 15.73%) mas esta diferença não é estatisticamente significativa (*p-value* = 0.175). A análise multivariada não revelou uma relação independente entre a área relativa de cálcio nas placas de ateroma com os níveis séricos de HDL e terapêutica com estatinas, na presença de osteoporose, tendo em conta a idade e o sexo.

Palavras-chave: aterosclerose; osteoporose; calcificação vascular; cálcio

ABSTRACT

The relationship between osteoporosis (OP) and atherosclerosis is still unclear. Despite the epidemiological association and the existence of common risk factors such as the influence of age in both pathologies, there are clinical, genetic and pathogenic data, suggesting the possibility of a common pathway. Vascular calcification has been an important target of investigation. There are several studies that demonstrate increased vascular calcifications in patients with OP.

The present study aimed to histologically evaluate the calcium deposits in atherosclerotic plaques of patients with and without osteoporosis, through a semi-quantitative analysis of calcium deposits.

In this study were included 32 patients undergoing elective carotid endarterectomy, from which were obtained blood samples and the atheroma plaque. A dual-energy X-ray absorptiometry (DXA) was performed and a structured clinical protocol was applied.

Samples were stained with *Alizarin Red S* and scanned with *NanoZoomer®*, in order to observe the calcium deposits within the atherosclerotic plaques. The semiquantitative analysis by *ImageJ®* showed numerically higher median values of relative calcium area in the atheroma plaques from osteoporotic patients (median: 45.46%) comparing to patients with normal bone mineral density (median: 15.73%), although this difference was not statistically significant (*p-value* = 0.175). In multivariate analysis, we do not found an independent relationship, between the relative area of calcium in atherosclerotic plaques with serum HDL levels and statins therapy, in the presence of osteoporosis, adjusted to gender and age.

Key-words: atherosclerosis; osteoporosis; vascular calcification; calcium

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Introduction

Atherosclerosis is a chronic inflammatory disease highly prevalent in developed countries, despite the advances in medical diagnosis and therapeutics. There are numerous modifiable risk factors for atherosclerosis development, such as hypertension, diabetes, obesity, smoking and hypercholesterolemia. It preferentially affects large and medium size arteries in regions with special susceptibility of arterial ramifications for atherogenesis. [1, 2, 3] These locations, namely the bifurcation of the common carotid artery, are associated with blood flow turbulence. [4, 5, 6]

Atherosclerosis manifests pathologically through the formation of atheroma plaques, a process called atherogenesis [1, 2] Atherogenesis is a complex process whose first macroscopic signs are lipid streaks in the arterial wall that progress, usually over several years, from intimal thickening (American Heart Association (AHA) Type I lesion) towards the formation of fibroatheroma (AHA Type IV lesion). [2] This is possible through migration of circulating lipoproteins, especially cholesterol-containing low-density lipoproteins (LDL), into the subendothelial space and their interaction with extracellular matrix components. [1, 2, 7, 8] Furthermore, since the beginning of atherosclerotic lesions, this process involves oxidative mechanisms during the lipoprotein sequestration below the intima layer. These modified lipoproteins trigger an inflammatory response which results in leukocyte signaling and recruitment. Circulating monocytes start expressing adhesion molecules, proteases and selectins, which promote diapedesis. [1, 2, 7, 8] Then, in the extravascular space, macrophages undergo mitosis and intensify the expression of lipoproteins' modifying receptors to phagocytize the oxidative lipoproteins, transforming into foam cells. The lipid streak may evolve into a more complex atheroma plaque through smooth muscular cells migration from the arteries media. [1, 2, 7, 8, 9]

Remarkably, plaque growth has an intermittent pattern with inactivity periods alternating with hyperactivity phases. Also, there is evidence that not all lipid streaks evolve into a complex atheroma plaque. [1, 10, 11] The progression of this process depends on an intricate balance between lipoproteins integrations, leucocytes activity, extracellular matrix remodeling, apoptosis and mitoses of cellular components of the plaque. Moreover, mainly in later stages, fibrosis, neovascularization and calcification of the atherosclerotic plaque may occur. [1, 2, 10, 11]

Intimal atherosclerotic calcification is considered the most common form of calcific vasculopathy. [12] Atherosclerotic calcification was first recognized in the 19th century and believed to be a passive, degenerative phenomenon. However, over the last two decades, vascular calcification has been recognized as an active and regulated process. There are four non-exclusive mechanisms that explain the calcification process in the arterial wall: dead macrophages and vascular smooth muscle cells (VSMC) release apoptotic fragments which may serve as a nucleate apatite deposition [12, 13, 14]; nuclear circulating complexes released from bone remodeling or matrix vesicles locally released by macrophages and VSMC [14]; decreased tissue-derived mineralization inhibitors [12, 13, 14]; and, on the other hand, subpopulations of vascular cells which might undergo osteogenic differentiation, stimulated by modified lipoproteins, advanced lipid peroxidation end products (ALEs) and advanced glycation end products (AGEs), possibly resulting in a dynamic and controlled process, similar to bone development and metabolism. [9, 12, 13, 14]

Endochondral ossification has been assumed as a probable mechanism for bone development in atherosclerotic lesions. Throughout the de-differentiation of VSMC, losing their expression of smooth muscle-specific markers, chondroblasts might arise and induce hyaline cartilage' formation. The osteochondral cells differentiate into osteoblasts, which are responsible for bone formation. [9, 12, 14, 15] Moreover, atherosclerosis pathophysiology involves macrophages, which have been reported to undergo osteoclastic differentiation, when exposed to particulate calcium mineral. In contrast, few studies, refute any kind of evidence for significant expression of chondrogenic markers in human atherosclerotic lesions, as collagen type II or X, S-100 protein and transcription factor SOX9, or evidence of endochondral ossification. [16, 17, 18]

Cardiovascular diseases are considered risks factors for the development of numerous diseases, including osteoporosis (OP) [19, 20]. This pathology is the most common metabolic bone disease, characterized by decreased bone mineral density (BMD) due to trabecular microstructure deterioration with consequent increased bone fracture risk. Bone is largely composed of hydroxyapatite crystals, organic component (collagen and proteoglycans, among others) and water. Electron microscopy studies have shown that hydroxyapatite crystals are arranged along the matrix and its components, phosphorus and calcium, are derived from nutritional reserves of the organism. [21, 22] It is known that calcium deficit plays a key role in OP pathophysiology, through the excessive

secretion parathyroid hormone and, consequently, severe bone resorption. The underlying pathophysiology lies in the imbalance between bone formation and resorption. [23, 24]

The link between OP and atherosclerosis is still to be clearly established. [20] In addition to the epidemiological relationship and the influence of age in both diseases [25], there is a range of common elements which assured a relationship among this two pathologies. [20, 26] This includes the existence of multiple common risk factors [27 - 32] (age, smoking, low physical activity, excessive alcohol intake, hormonal state, lipid and calcium intake) and possible common pathophysiological mechanisms (inflammation[31], homocysteine levels [34, 35], vitamin D [36, 38], lipid metabolism [31], hypertension [38, 39] and hormonal impact [40, 41]) and genetic sharing (osteoprotegerin and matrix GIa protein genes) [6, 29]. Furthermore, there is evidence of increased bone remodeling and, consequently, bone mass decrease in patients with advanced atherosclerotic disease. Still, it is uncertain if arterial calcification results in decrease bone mass or it is only a consequence of a shared pathophysiologic mechanism. [25, 42, 43, 44]

Several studies have shown an increase of calcium deposits in atherosclerotic plaques of patients with OP. However, most of these works used computed tomography [11, 43, 45, 46], X-rays imaging or intravascular ultrasonography. [11, 47, 48] Since there is no comparative histological data available between patients with and without OP and given the association between these two diseases, our hypothesis is that calcium deposits in atheroma plaques are greater in patients with decreased BMD. As such, the aim of this study was to histologically identify and quantify calcium deposits within atherosclerotic plaques and compare its extent between patients with and without OP. Moreover, we aim to correlate the extent of calcium deposits in atherosclerotic plaques with patient's clinical characteristics.

Materials and Methods

1. Patients

We included patients from the Vascular Surgery Department of Centro Hospitalar Lisboa Norte, EPE - Hospital de Santa Maria who underwent carotid endarterectomy. All patients gave their informed consent for collection and analysis of biological samples (blood and atheroma plaque) and to perform a bone densitometry (Annexes I and II, respectively).

Information about cardiovascular risk factors (gender, age, hypertension, smoking, alcohol intake, diet, diabetes mellitus, dyslipidemia, physical activity, height and weight), personal and family history of relevant diseases (rheumatologic, cardiovascular, neurovascular, endocrine and others), personal and family history of fractures, as well as, medication in the last 3 months was acquired just prior to surgery through a structured clinical protocol (Annex III). A fasting blood sample was obtained at the time of the surgery.

Bone mineral density ($g.cm^{-2}$) was evaluated by dual-energy X-ray absorptiometry (DXA) of the lumbar spine and hip. The number of standard deviations in relation to the peak bone density of sex-matched adult population (*t-score*) was also calculated. [21] Patients were classified according to the World Health Organization (WHO) in Table 1.

Table 1 – WHO *t-score* classification[21]

Classification	*t-score*
Normal	> -1.0
Osteopenia	-1.0 ≤ and ≥ -2.5
Osteoporosis	< -2.5

We divided patients in two categories, excluding the "osteopenia" cluster from Table 1, in order to have two groups, with 16 patients each, representing the t-score edges:

- Group A (Normal BMD, *t-score* > -1.0);
- Group B (Osteoporosis, *t-score* < -2.5).

2. Plaque Processing

The atheroma plaques were carefully dissected and immediately transferred to the Rheumatology Research Unit' laboratory. Upon arrival, plaques were subdivided, frozen in OCT (Optimal Cutting Temperature compound) and then stored at -80ºC until processing.

Frozen plaques were sectioned crosswise over their longitudinal axis using a cryostat, and the plaques major segment was used for histological studying.

For calcium study, *Alizarin Red S* staining (pH: 4.11) was used. This stain is an anthraquinone derivative used to histologically detect the presence of calcium, producing orange-red staining. Elements such as magnesium, manganese, barium, strontium and iron may interfere with the staining process. [49] However, the chemical composition of the atheroma plaque is scarce in these elements to restrict the reaction. [49, 50] A mouse paw was used as a positive control (Annex IV).

3. Semi-Quantitative and Statistical Analysis

After staining, using *NanoZoomer*®, it was possible to visualize and scan the histological slides (Resolution 10X). Then, with the *Image J*® program, we scored the total area of atheroma plaque and the stained areas with *Alizarin Red S*¸ in each slides. The relative area of stained calcium was calculated aiming to perform a semi-quantitative analysis of calcium in atherosclerotic plaques and a comparison between groups.

A descriptive analysis of patient's data and comparison of the aforementioned groups by *T-test* or *Mann-Whitney Test*, as appropriate according to the normality tests results, was performed. We used the *SPSS*® *software V23*® for the statistical analysis.

4. Ethical Considerations

This project meets the standards of the WMA Declaration of Helsinki (Seoul 2008 revision), and was submitted to and approved by the Ethics Committee of the Hospital de Santa Maria. Patients were requested to give their written informed consent for participation in the study, collection of biological samples and for the realization of DXA (Annexes I and II).

Results

1. Population Characterization

This study took place between October 2014 and July 2015 and 32 patients with carotid atherosclerosis who underwent carotid endarterectomy in the Vascular Surgery Department of Hospital de Santa Maria were included. Their demographical and clinical characteristics are resumed in Table 2.

Table 2 – Baseline characteristics of the Patients

Variable	Results
Demographic	**N = 32**
Age – mean ± SD (median) *years*	71.59 ± 8.63 (74)
Male sex – no. (%)	16 (50)
Clinical	
Daily Alcohol Intake – no. (%)	
< 3 units per day	25 (78.1)
> 3 units per day	7 (21.9)
Smoking Status – no. (%)	
Current smoker	4 (12.5)
Former smoker	12 (37.5)
Coexisting conditions – no. (%)	
Hypertension	26 (81.3)
Diabetes mellitus	13 (40.6)
Dyslipidemia	25 (78.1)
Previous Stroke	20 (62.5)
Metabolic Bone Diseases (MBD) – no. (%)	
Primary OP	16 (50)
Other MBD	0 (0)
Previous fractures[1]	16 (50)
Medical Therapy – no. (%)	
Anti-hypertensive medication[2]	28 (87.5)
Statins therapy	20 (62.5)
Other lipid lowering therapies[3]	6 (8.8)
Oral anti-diabetics therapy	11 (34.4)
ASA therapy	23 (71.9)
Calcium supplement	2 (6.3)
Vitamin D supplement	1 (3.1)
Histology	
Calcium Relative Area (%) – median [min; max]	17.79 [0.03; 90.36]

SD – standard deviation; OP – osteoporosis; ASA – acetylsalicylic acid.

[1] Includes fragility and non-fragility fractures;
[2] Includes diuretics, calcium channel blockers, ACE inhibitors, ARBs, vasodilators and beta blockers;
[3] Includes fibrates, niacin, bile-acid resins and cholesterol absorption inhibitors.

Taking into account the WHO classification of body mass index (BMI), 12 patients (38.5%) are classified as overweight and 9 patients (34.1%) as obese. Moreover, 21 patients (65.6%) have a sedentary lifestyle and only 12 patients (37.5%) were previously physically active. Most patients have familiar history of cardiovascular disease (58.3%) and an estimated 10-year risk for cardiovascular events of 2.78%.

As previously stated, half of the population, has primary OP (mean femoral neck (FN) t-score -2.91 ± 0.20) and the other 50% had normal BMD (mean FN *t-score* -0.36 ± 0.85). Only 5 patients (15.6%) have familiar history of OP. As calculated by the WHO Fracture Risk Assessment Tool (FRAX®), our population has a 9.04% probability of having a major fracture in the next 10 years.

Table 3 shows patient's serum measurements.

Table 3 – Fasting mean serum levels

Variable	Mean ± SD	Reference values
Total cholesterol	148.75 ± 38.07	< 190.0 mg/dL
LDL cholesterol	90.47 ± 30.43	< 110 mg/dL
HDL cholesterol	37.47 ± 12.01	> 40 mg/dL
Triglycerides	104,78 ± 69.64	< 150 mg/dL
Homocysteine	17.16 ± 4.65	5.0 – 12.0 mg/dL
Uric Acid	5.50 ± 1.78	3.1 – 7.8 mg/dL
Calcium	8.9 ± 0.85	8.6 – 10.2 mg/dL
25(OH)D	18.92 ± 10.68	≥ 30.0 mg/dL

Reference values defined by the Clinical Pathology Laboratory of Hospital de Santa Maria

SD – standard deviation; LDL – Low-density lipoprotein; HDL – High-density lipoprotein; 25(OH)D – 25-hydroxy vitamin D.

1.1. Subgroup Analysis – Diagnosis of Osteoporosis

A subgroup analysis according to the diagnosis of OP was performed and the results are presented in Table 4 and Figure 1.

Table 4 – Subgroup analysis: Osteoporosis

Variable	Normal	OP	*p-value*
	N = 16	N = 16	
Calcium Relative Area – % Median [Min; Max]	**15.73 [0.03;82.50]**	**45.46 [2.22; 90.36]**	**0.175[a]**
Mean Age - *years*	69.31 ± 9.45	73.88 ± 7.31	0.234[a]
Gender			1.000[b]
Male – N (%)	8 (50)	8 (50)	
Female – N (%)	8 (50)	8 (50)	
Current and former smokers – N (%)	6 (37.5)	10 (62.5)	0.157[b]
> 3 alcohol units intake day – N (%)	11 (68.8)	14 (87.5)	0.394[c]
Hypertension – N (%)	14 (87.5)	12 (75)	0.654[c]
Diabetes – N (%)	5 (31.3)	8 (50)	0.280[b]
Dyslipidemia – N (%)	12 (75)	13 (81.3)	1.000[c]
Previous Stroke – N (%)	10 (62.5)	10 (62.5)	1.000[b]
Calcium serum levels – mg/dL	9.07 ± 0.622	8.74 ± 1.02	0.267[a]
Vitamin D serum levels – mg/dL	15.81 ± 7.20	22.02 ± 12.78	0.181[a]
FN *t-score*	-0.36 ± 0.85	-2.91 ± 0.20	< 0.001[a]
10-year risk of major fracture – %	4.67 ± 3.07	13.42 ± 12.42	< 0.001[a]
10-year risk of CV events – %	2.93 ± 1.73	2.63 ± 1.20	0.556[a]

OP – osteoporosis; FN BMD – femoral neck bone mass densitometry; CV - cardiovascular
[a] According to *Mann-Whitney Test* [b] According to *Chi-Square Test* [c] According to *Fisher's Exact Test*

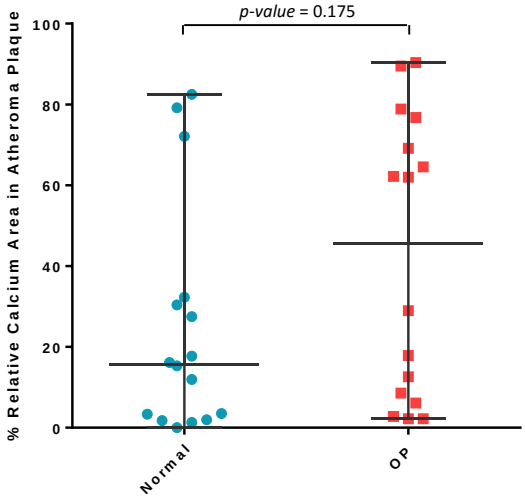

Figure 1 – Relative calcium area in the atheroma plaque from patients with normal BMD and with OP

There is no statistically significant difference between the relative calcium area of atheroma plaques from patients with and without OP, although the OP cluster has median calcium relative areas numerically higher than the normal group (*p-value* = 0.175). As expected, the OP cluster has higher 10-year risk of major fracture (13.42 %) than patients without OP (4.67 %) and this difference is statistically significant (*p-value* < 0.001).

1.2. Subgroup Analysis – Gender

According to the gender subgroup analysis (Table 5), there are more current and former smokers' male than female patients. Also, the male group has a significantly higher risk of cardiovascular events in 10 years than the female group. There is no statistically significant difference in calcium relative area between genders (Figure 2, *p-value* = 0.132), although women have a higher median value than men.

Table 5 – Subgroup analysis: Gender

Variable	Male	Female	*p-value*
	N = 16	N = 16	
Calcium Relative Area – % Median [Min; Max]	**15.73 [0.03;89.48]**	**29.67 [1.99; 90.36]**	**0.132[a]**
Mean age - *years*	73.38 ± 7.54	69.81 ± 9.50	0.157[a]
Current and former smokers – N (%)	16 (100)	5 (31.3)	0.034[b]
> 3 alcohol units intake day – N (%)	7 (43.8)	0 (0)	0.007[c]
Hypertension – N (%)	14 (87.5)	12 (75)	0.654[c]
Diabetes – N (%)	5 (31.3)	8 (50)	0.280[b]
Dyslipidemia – N (%)	11 (68.8)	14 (87.5)	0.394[c]
Previous Stroke – N (%)	11 (68.8)	9 (56.3)	0.465[b]
Calcium serum levels – mg/dL	8.87 ± 1.00	8.93 ± 0.70	0.812[a]
Vitamin D serum levels – mg/dL	18.81 ± 9.04	19.03 ± 17.20	0.792[a]
FN *t-score*	-1.58 ± 1.41	-1.68 ± 1.50	0.472[a]
10-year risk of major fracture – %	7.76 ± 6.30	10.31 ± 12.70	0.497[a]
10-year risk of CV events – %	3.31 ± 1.45	2.25 ± 1.34	0.022[a]

FN BMD – femoral neck bone mineral density; CV – Cardiovascular
[a] According to *Mann-Whitney Test* [b] According to *Chi-Square Test* [c] According to *Fisher's Exact Test*

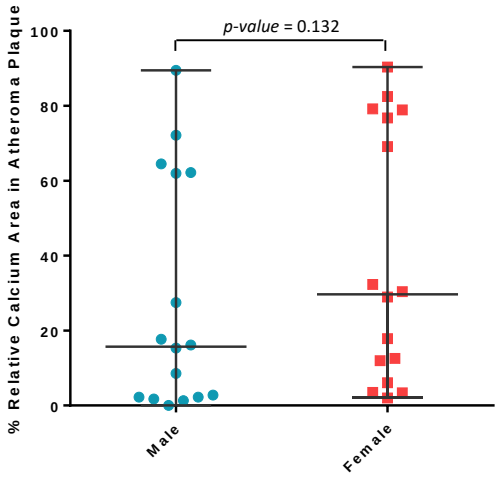

Figure 2 – Calcium relative area in the atheroma plaque by gender

1.3. Subgroup Analysis – Previous Stroke

Finally, a subgroup analysis according to the occurrence of a previous stroke was performed and the results are presented in Table 6 and Figure 3.

Table 6 – Subgroup Analysis: Stroke

Variable	Non Prev. Stroke	Previous Stroke	*p-value*
	N = 12	N = 20	
Calcium Relative Area – % Median [Min; Max]	**8.63 ± 0.74**	**9.05 ± 0.89**	**0.148[a]**
Mean Age - *years*	72.67 ± 9.64	70.95 ± 8.15	0.447[a]
Gender			0.467[b]
Male – N (%)	5 (41.7)	11 (55)	
Female – N (%)	7 (58.3)	9 (45)	
Current and former smokers – N (%)	3 (25)	13 (65)	0.028[b]
> 3 alcohol units intake day – N (%)	2 (16.7)	5 (25)	0.683[c]
Hypertension – N (%)	10 (83.3)	16 (80)	1.000[c]
Diabetes – N (%)	5 (41.7)	8 (40)	1.000[c]
Dyslipidemia – N (%)	10 (83.3)	15 (75)	0.683[c]
Previous Stroke – N (%)	12.27 [0.03; 82.50]	22.67 [1.28; 90.36]	0.668[a]
Calcium serum levels – mg/dL	21.32 ± 14.85	17.48 ± 7.26	0.876[a]
Vitamin D serum levels – mg/dL	6/12 (50)	10/20 (50)	1.000[b]
FN *t-score*	-1.84 ± 1.16	-1.51 ± 1.59	0.532[a]
10-year risk of major fracture – %	11.91 ± 14.73	7.32 ± 5.24	0.483[a]
10-year risk of CV events – %	2.25 ± 0.97	3.10 ± 1.65	0.216[a]

FN BMD – femoral neck bone mineral density; CV - Cardiovascular
[a] According to Mann-Whitney Test [b] According to Chi-Square Test [c] According to Fisher's Exact Test

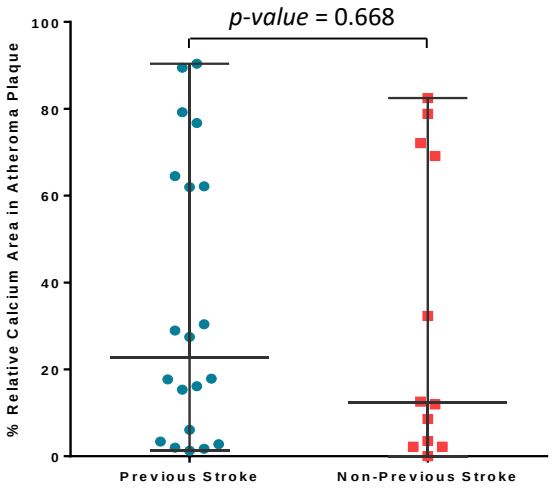

Figure 3 – Relative calcium area in the atheroma plaque in patients with and without previous stroke

We found no statistically significant difference between calcium relative area in the atheroma plaque in patients with or without previous stroke. Moreover, significantly more patients with previous stroke are current or former smokers, although this fact seems to have no effect on the 10-year risk of CV events probability.

2. Correlations

Throughout the clinical data and fasting blood samples, we correlate the calcium relative area with serum lipid parameters, the results are present in Table 7 and Figures 4 and 5.

Table 7 – Correlation between calcium relative area and serum lipid parameters (N = 32)

Variable	Total cholesterol	HDL cholesterol	LDL cholesterol	Triglycerides
Calcium Relative Area	$\rho = -0.523$ *p-value* $= 0.002$	$\rho = -0.549$ *p-value* $= 0.001$	$\rho = -0.299$ *p-value* $= 0.097$	$\rho = -0.213$ *p-value* $= 0.238$

ρ – Spearman's correlation coefficient
HDL – High-density lipoprotein; LDL – Low-density lipoprotein

The correlation between total cholesterol and relative calcium area ($\rho = -0.523$, *p-value* $= 0.002$), and the correlation between HDL cholesterol and relative calcium area ($\rho = -0.549$, *p-value* $= 0.001$) are statistically significant in the whole group. However, there is no statistically significant correlation between LDL serum cholesterol or triglycerides and relative calcium area in the atheroma plaque.

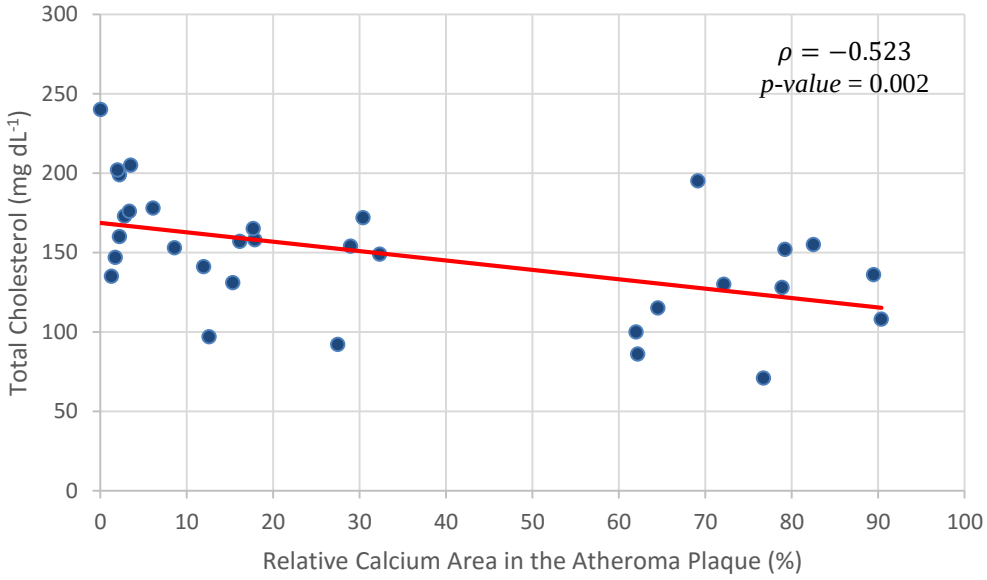

Figure 4 – Correlation between total cholesterol serum levels and relative calcium area

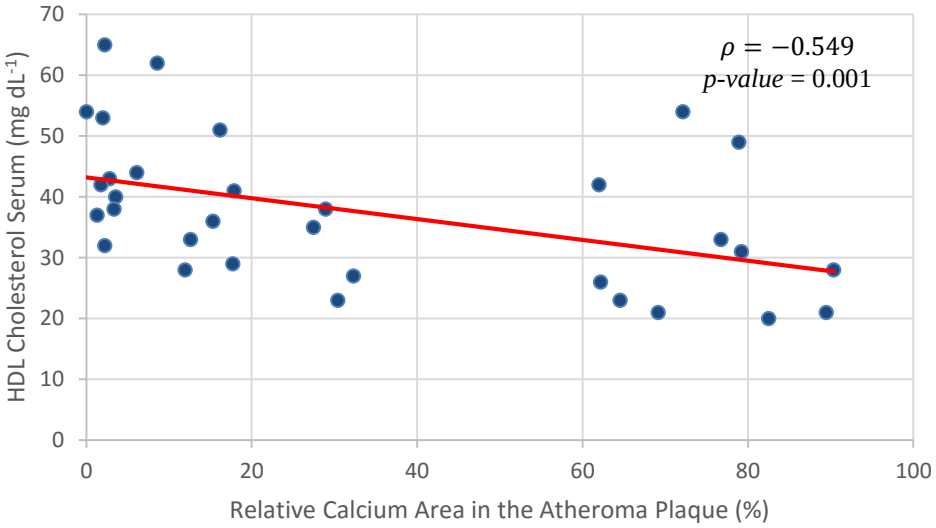

Figure 5 – Correlation between HDL cholesterol serum levels and relative calcium area

Then, we also correlate the calcium serum levels and vitamin D serum levels with the calcium relative area in the atheroma plaque, and the results are shown in Table 8.

Table 8 – Correlation between calcium relative area and, calcium and vitamin D serum (N = 32)

Variable	Calcium levels	25(OH)D levels
Calcium Relative Area	$\rho = -0.129$ *p-value* $= 0.487$	$\rho = 0.136$ *p-value* $= 0.459$

ρ – Spearman's correlation coefficient
25(OH)D – 25-hydroxy vitamin D

There is no statistically significant correlation between serum calcium and vitamin D with calcium relative area in the atheroma plaque.

Finally, we correlated lipid lowering therapies, namely statins therapy (Table 9), with the calcium relative area.

Table 9 – Correlation between statins therapy and calcium relative area.

Variable	Therapy		*p-value*
	Statins	Without statins	
Calcium Relative Area – % Median [Min; Max]	68.32 [0.03; 68.32]	13.96 [1.28; 90.36]	0.029[a]

[a] According to *Mann-Whitney Test*

Patients who are on statins have statistically significant large calcium areas than patients without statins therapy (68.32% vs 13.16%, *p-value* $= 0.029$), as determined by a U Mann-Whitney test. Other lipid lowering therapies do not seem to influence the atheroma plaques calcium relative area (*p-value* $= 0.412$).

3. Multivariate analysis

Aiming at predicting the independent association of some variables with the diagnosis of OP, a logistic regression was performed between OP diagnosis (dependent variable) and calcium relative area in the atheroma plaque, adjusted to age, gender, total cholesterol serum levels, HDL cholesterol serum levels and statins therapy (explanatory variables).

Age and gender were included in this analysis once they are common risk factors for the development of atherosclerosis and osteoporosis. [1, 2, 3, 19, 22] Additionally, there is a numerical difference on age between OP cluster (73.88 years) and normal cluster (69.31 years), even it is not statistically significant (*p-value* = 0.234).

Although there is no statistically significant difference between the relative calcium area of atheroma plaques from patients with and without metabolic bone disease (*p-value* = 0.175), the OP cluster has median calcium relative areas numerically higher (median: 45.46%) than the normal group (median: 15.73%). Thus, we included this variable in the analysis.

Furthermore, HDL cholesterol serum levels, total cholesterol serum levels and statins therapy were counted in this analysis because we found statistically significant correlation between this variables and the calcium relative area.

Once we only have 32 patients, we attended with four explanatory variables in each model. We used backward method for the analysis.

Table 10 - Model attending age, gender, calcium relative area in the atheroma plaque and HDL cholesterol serum levels.

Dependent Variable		Diagnose of Osteoporosis					
Independent Variables		B	S.E.	Wald	dF	Sig.	Exp(B)
1	**Age**	-0.050	0.053	0.902	1	0.342	0.951
	Gender (male)	0.284	0.788	0.130	1	0.718	1.329
	Calcium relative area	0.394	0.474	0.690	1	0.406	1.482
	HDL cholesterol levels	0.000	0.032	0.000	1	0.993	1.000
2	**Age**	-0.056	0.050	1.232	1	0.267	0.946
	Gender (male)	0.024	0.793	0.001	1	0.976	1.024
	Calcium relative area	-0.014	0.013	1.270	1	0.260	0.986

3	**Age**	-0.055	0.049	1.296	1	0.255	0.946
	Calcium relative area	-0.014	0.012	1.347	1	0.241	0.986
4	**Calcium relative area**	-0.018	0.012	2.269	1	0.132	0.982

Model Summary	**-2 Log likelihood**	**Cox & Snell R Square**	**Nagelkerke R Square**
1	40.686	0.070	0.094
2	40.547	0.112	0.150
3	40.548	0.112	0.150
4	41.938	0.073	0.097

S.E. – Standard error; dF – degrees of freedom;
HDL – High-density lipoprotein

Table 11 - Model attending age, gender, calcium relative area in the atheroma plaque and total cholesterol serum levels.

Dependent Variable	**Diagnose of Osteoporosis**					
Independent Variables	**B**	**S.E.**	**Wald**	**dF**	**Sig.**	**Exp(B)**
1 **Age**	-0.039	0.054	0.523	1	0.470	0.962
Gender (Male)	0.398	0.789	0.254	1	0.614	1.489
Calcium relative area	0.255	0.512	0.248	1	0.619	1.290
Total cholesterol levels	0.015	0.012	1.557	1	0.212	1.015
2 **Age**	-0.061	0.048	1.634	1	0.201	0.941
Gender (male)	0.358	0.787	0.207	1	0.649	1.431
Total cholesterol levels	0.015	0.011	1.742	1	0.187	1.015
3 **Age**	-0.057	0.047	1.471	1	0.255	0.944
Total cholesterol	0.014	0.011	1.678	1	0.195	1.014
4 **Total cholesterol**	0.016	0.011	2.350	1	0.125	1.016

Model Summary	**-2 Log likelihood**	**Cox & Snell R Square**	**Nagelkerke R Square**
1	38.989	0.120	0.160
2	39.927	0.129	0.173
3	40.137	0.124	0.165
4	41.715	0.079	0.106

S.E. – Standard error; dF – degrees of freedom;

Table 12 - Model attending age, gender, calcium relative area in the atheroma plaque and statins therapy.

Dependent Variable		Diagnose of Osteoporosis					
Independent Variables		B	S.E.	Wald	dF	Sig.	Exp(B)
1	Age	-0.052	0.055	0.912	1	0.340	0.949
	Gender (male)	0.279	0.760	0.135	1	0.713	1.322
	Calcium relative area	0.424	0.519	0.666	1	0.415	1.528
	Statins (no therapy)	-0.127	0.888	0.020	1	0.888	1.883
2	Age	-0.056	0.050	1.232	1	0.267	0.946
	Gender (male)	0.024	0.793	0.001	1	0.976	1.024
	Calcium relative area	-0.014	0.013	1.270	1	0.260	0.986
3	Age	-0.055	0.049	1.296	1	0.255	0.946
	Calcium relative area	-0.014	0.012	1.347	1	0.241	0.986
4	Calcium relative area	-0.018	0.012	2.269	1	0.132	0.982

Model Summary	-2 Log likelihood	Cox & Snell R Square	Nagelkerke R Square
1	40.666	0.071	0.094
2	40.547	0.112	0.150
3	40.548	0.112	0.150
4	41.938	0.073	0.097

S.E. – Standard error; dF – degrees of freedom;

Discussion

Both cardiovascular disease and OP account for most of the morbidity and mortality in our elderly population despite significant improvements in medical treatments. [4, 5, 6] Growing evidence indicates the existence of a correlation between cardiovascular diseases and OP, regardless of age. Studies have highlighted the presence of vascular calcification in patients with advanced atherosclerosis and decreased BMD, especially in women. [24, 42, 43, 44] The present study aimed to compare the histological calcium content in the atheroma plaques of OP and non-OP patients with advanced carotid atherosclerosis.

Ultrasound intima-medial thickness, intravascular ultrasonography, electron-beam computed tomography and x-ray studies [11, 42, 45-48] have identified consistently, inverse associations between BMD and calcium deposits in the plaque. However, there were no histopathological studies before ours that compare the amount of calcium within the plaque between these two groups of patients. Our study did not found a statistical significant association between the histological relative amount of calcium within the atheroma plaque in patients with and without OP. These results do not support our hypothesis and are not in agreement with the results already described in humans using x-rays or CT. It is important to emphasize that our sample size is relatively small, with 16 patients per group, what is probably underpowered. Furthermore, we only focused on the stained area with Alizarin Red S, not attending to the different stain gradations of the plaques that could indicate a more realistic result on the amount of calcium deposits per plaque area. Additionally, we are only studying the plaques major segment and, probably, calcium deposits are not distributed evenly.

We do not have an independent association between HDL cholesterol serum and the calcium relative area in the plaque, apart from age, gender and OP. Though, there is accumulating evidence that HDL has a protective role in cardiovascular disease pathophysiology [51, 52, 53] Parhami F, *et al* (2002) showed, in vitro, that HDL blocks the osteogenic differentiation of calcifying vascular cells induced by oxidized HDL and cytokines (Interleukin (IL)-1β and IL-6). [51] Moreover, these studies concluded that HDL regulates the early and late stages of osteogenic differentiation. In fact, these cells, when exposed to HDL, have a significantly lower alkaline phosphatase activity, an important marker of osteoblastic differentiation in osteoprogenitor cells. In addition, HDL also

inhibits matrix calcification in vascular cell cultures, a marker in later phases of osteogenic differentiation. Overall, these results suggest that HDL might regulate vascular calcification by directly inhibiting osteogenic differentiation. [51, 52, 53]

Studies have increasingly shown evidence indicating that not all types of plaque calcification have the same clinical outcome in terms of plaque vulnerability. On the one hand, microcalcifications within the fibrous cap of the atheroma plaque are associated with substantial stress accumulation within the cap that might cause subsequent plaque rupture. Also, macrocalcifications that develop beneath a thick fibrous cap are responsible for a more stable vascular atherosclerotic plaque, with less blood flow turbulence. [12, 13, 15, 54, 55] One more limitation of this study is that we cannot understand what type of vascular calcification these patients have, because alizarin red only stains calcium deposits and do not differentiate between microcalcification and bone-like tissue.

Furthermore, we did not find an independent association between statins therapy and a higher amount of calcium in the atherosclerotic plaque, apart from the influence of gender, age and OP. However, previous studies showed that, in spite of diminishing the volume of the atheroma plaque, statins actually promote vascular calcification.[53, 55, 56] Lenglet *et al* (2014) have already demonstrated that the intake of statins was linked to increase OPG expression in atheroma plaques. [57] Nevertheless, these studies usually use patients on high-intensity statin therapy. Despite having advanced atherosclerotic disease, most of our patients were on low-intensity statin therapy and, to our knowledge, there are no previous studies about vascular calcification in patients with carotid atherosclerosis on low-intensity statin therapy.

Characterizing the calcium content in the atheroma plaque and understanding its relationship with BMD can make a way for the identification of new pathophysiological mechanisms. Additional studies might be useful to highlight the role of preventive and therapeutic measures, as well as to establish a more accurate prognostic evaluation of bone and vascular disease.

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ANNEX I

Biological Samples Informed Consent

INFORMAÇÃO AO DADOR DE AMOSTRAS BIOLÓGICAS

Título do projecto de investigação

Biobanco do Instituto de Medicina Molecular (IMM) da Faculdade de Medicina da Universidade de Lisboa - Banco de amostras biológicas Humanas para fins de investigação biomédica

Objectivo do Estudo

A criação de um banco de amostras biológicas humanas permitirá o desenvolvimento de ferramentas de diagnóstico e investigação em múltiplas áreas da medicina, com especial impacto em doenças crónicas, como as doenças oncológicas, cardiovasculares, neurológicas, ósseas e imunológicas. Contudo, este objectivo só será cumprido com a colaboração dos doentes e de indivíduos saudáveis, através da doação de amostras biológicas que serão guardadas e preservadas em condições apropriadas de forma a serem utilizadas para futuros estudos. Caso o doente ou indivíduo saudável e/ou o seu representante legal decida participar, terá de fazer apenas os procedimentos habituais de uma consulta.

Procedimentos

No caso de concordar em participar neste projecto, ser-lhe-á colhida uma amostra biológica. A amostra habitualmente solicitada será aproveitada a partir da colheita de sangue e/ou urina que irá efetuar. Para os indivíduos que estejam a realizar exames diagnósticos ou que estejam a ser sujeitos a tratamentos cirúrgicos poderá ser pedida autorização para colheita de uma pequena amostra do material removido durante o procedimento (como por exemplo saliva, liquido cefalo-raquidiano, tecidos removidos para biópsias ou removidos no decurso de cirurgias). Estas colheitas serão efetuadas sem alterar os procedimentos médicos habituais e sem interferir com a rentabilidade diagnóstica do procedimento ou com o sucesso da cirurgia. . Esta amostra será preservada em condições apropriadas e as informações clínicas com ela relacionada serão introduzidas numa base de dados, passando a sua identificação pessoal a estar codificada e não acessível aos utilizadores das amostras.

A doação da amostra é voluntária e revogável, sendo que o dador, ou o seu representante legal, tem o direito de retirar a amostra e/ou interromper a colaboração assim que achar conveniente, sem necessidade de justificação e não podendo ser descriminado por isso. O dador ou o seu representante legal deverá manifestar por escrito a sua vontade em retirar a amostra ou interromper a colaboração e nestas situações a amostra será imediatamente destruída.

O Biobanco do IMM propõe-se armazenar as amostras biológicas e seus possíveis derivados tais como, soro, plasma, DNA, RNA e células. No caso da colheita de sangue ou em qualquer outra circunstância de colheita para a qual seja necessário um acto médico invasivo será adoptada uma técnica de imortalização de células, evitando-se assim nova colheita de amostra.

O biobanco do IMM não divulgará resultados envolvendo o material biológico. No entanto, o dador poderá escolher se quer ser informado dos resultados com potencial relevância para a sua saúde. O pedido de resultados deverá ser feito por escrito para o Biobanco do IMM pelo dador ou representante legal e deve ser expresso no consentimento informado.

Serão cumpridas todas as normas éticas aceites internacionalmente para o uso de matérias biológicos para fins de investigação. Todos os projetos que fizerem uso das amostras depositadas no Biobanco do IMM serão submetidos à Comissão de Ética competente para a sua avaliação.

Identificação das amostras e Confidencialidade

A existência de um biobanco pressupõe a existência de uma base de dados contendo informação clínica referente ao doente ou indivíduo saudável. Após a colheita, as amostras serão identificadas por um código de forma a preservar a privacidade.

Durante o desenvolvimento de um projecto de investigação, a equipa de investigação poderá ter necessidade de recolher informação do processo clínico para a execução do estudo. O anonimato será, contudo mantido, ou seja os dados constantes do seu processo clínico serão fornecidos ao investigador, mas sem qualquer identificação, ou qualquer informação que permita saber a quem pertencem.

A descodificação apenas poderá ser efectuada pelo médico (que será o responsável pela base de dados, de acordo com a informação fornecida à Comissão Nacional de Protecção de Dados - CNPD), em caso de absoluta necessidade, por motivos de saúde do dador e a pedido deste, e sempre de acordo com as disposições legais em vigor.

Os dados serão tratados confidencialmente, de acordo com a Lei, com os regulamentos e de acordo com as normas éticas aprovadas pela Comissão de Ética do CHLN/FMUL e pela CNPD.

Os dados resultantes dos estudos realizados serão alvo de publicação de uma forma anónima e agregada, em termos de percentagens ou de dados numéricos, nunca individualmente.

Tempo de conservação

As amostras serão conservadas por um período de 20 anos no Biobanco do Instituto de Medicina Molecular (IMM) da Faculdade de Medicina da Universidade de Lisboa, sob a responsabilidade da Equipa ligada ao projecto, enquanto este estiver devidamente credenciado pelas entidades competentes enquanto este estiver devidamente credenciado pelas entidades competentes. As coleções de amostras serão avaliadas periodicamente, nomeadamente para aferir da sua qualidade, podendo ser destruídas ou, findo o período da conservação, poder-se-á solicitar a prorrogação da conservação. Nestas condições excecionais o Biobanco do IMM poderá recontactar os dadores.

Comunicação e divulgação de dados

Os dados genéticos e as amostras biológicas colhidas para fins de investigação científica podem ser transferidos para outras organizações ou centros de investigação, para fins de pesquisa e somente em projetos desenvolvidos conjuntamente com o IMM, mediante consentimento do participante expresso na declaração de consentimento informado.

Possíveis Benefícios para os Participantes

Esta é uma doação altruísta, não havendo por isso qualquer compensação para o dador. Não se garante que este estudo envolva quaisquer benefícios directos para o participante. Se algum dos estudos puder ser relevante para a saúde do dador, este será informado, se essa for a sua vontade expressa na declaração de consentimento informado. Contudo, a sua participação proporcionará a aquisição de conhecimentos que poderão vir a beneficiá-lo a si ou a terceiros no futuro.

Riscos físicos previsíveis

Na maioria dos casos, os riscos e o desconforto associados serão mínimos ou inexistentes. Nas colheitas associadas a procedimentos com fins diagnósticos ou terapêuticos, os riscos e o desconforto serão os inerentes ao procedimento em si. Em qualquer dos casos, o dador será sempre antecipadamente informado dos riscos e grau de desconforto associados aos procedimentos.

Participação Voluntária e Direitos de Abandono

O presumível dador terá toda a liberdade para se recusar a participar no estudo ou retirar o seu consentimento, suspendendo a participação em qualquer momento e, consequentemente, as amostras serão destruídas. A participação é voluntária e a sua recusa em participar não envolverá qualquer penalização ou perda de benefícios. A recusa ou abandono não colocarão em risco o direito a receber tratamento ou assistência médica, presentemente ou no futuro.

O dador poderá retirar o seu consentimento nas modalidades **sem contacto futuro** (as amostras poderão ser usadas normalmente até se esgotarem, mas não serão estabelecidos futuros contactos para a obtenção de mais amostras) ou **sem uso futuro** (não serão estabelecidos futuros contactos e as amostras serão imediatamente destruidas e os registos eliminados).

Se tiver qualquer dúvida, em qualquer momento, mesmo após a colheita, sobre este estudo poderá contactar o Director do Biobanco do IMM:

Prof. Doutor Sérgio Dias e Doutor Joaquim Polido Pereira, dirigindo-se a:
Drª Angela Maria Afonso
Biobanco
Instituto de Medicina Molecular
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DECLARAÇÃO DE CONSENTIMENTO INFORMADO

Banco de amostras biológicas para fins de investigação biomédica

Investigador:_____ Hospital: _____
Nome do dador: _____
Número de estudo do dador: _____

Eu, , portador do bilhete de identidade/cartão do cidadão n.º [ ], declaro ter tomado conhecimento e aceitar participar neste projecto, de forma a contribuir para a criação de um banco de amostras biológicas com informação clínica associada, para fins de investigação biomédica.
Aceito que a minha amostra biológica seja utilizada em projectos de investigação de mecanismos das doenças, diagnóstico precoce, fatores de prognóstico e novos alvos terapêuticos em múltiplas áreas da medicina, nomeadamente nas doenças oncológicas, cardiovasculares, neurológicas, ósseas e imunológicas. Poderei revogar a autorização para utilização da minha amostra biológica e informação clínica em qualquer altura.
O objectivo do banco de amostras biológicas foi-me claramente explicado e foi-me dada a oportunidade de colocar questões sobre o seu funcionamento, bem como os procedimentos relativos à colheita e utilização da minha amostra biológica e dados a ela associados.
Declaro que aceito participar, voluntariamente, neste estudo. Especificamente concordo com os seguintes pontos:

- Consinto a colheita de material biológico (sangue / ..... / .....) e autorizo a conservação de amostras no Biobanco, de modo a que possam ser usados para pesquisas futuras, incluindo estudos genéticos e cultura de linhas celulares por investigadores portugueses e estrangeiros, sem fins lucrativos;

Sim ☐ Não ☐

- **Esta opção é para ser respondida apenas por participantes que já cederam amostras biológicas colhidas no âmbito de outros projetos.** Nestas circunstâncias, autorizo a transferência para o Biobanco as minhas amostras biológicas, préviamente colhidas no âmbito de outros projectos, de modo que elas possam ser utilizadas em pesquisas futuras, incluindo estudos genéticos e cultura de linhas celulares por investigadores portugueses e estrangeiros, mas sem fins lucrativos;

Sim ☐ Não ☐

- Estou consciente de que minha participação é voluntária e que posso em qualquer altura solicitar a destruição das minhas amostras biológicas, invalidando assim o consentimento informado prévio, sem justificar, tendo recebido a garantia de que o meu pedido não desenvolverá discriminação;

Sim ☐ Não ☐

- Declaro que quero conhecer resultados que possam ser relevantes para a minha saúde.

Sim ☐ Não ☐

- Autorizo ser contactado novamente pelo Biobanco do IMM para pedido de atualização sobre a minha situação clínica;

Sim ☐ Não ☐

_____ _____

Data Assinatura do Dador/Representante Legal

Em caso de representante legal, este actua na qualidade de:

□ Titular do poder paternal, quando o dador é menor
□ Tutor, quando o dador foi declarado interdito
□ Herdeiro, quando o dador faleceu

Discuti este estudo de investigação com o participante e/ou o seu representante legal, utilizando uma linguagem compreensível e apropriada. Informei adequadamente o participante sobre a natureza deste estudo e sobre os seus possíveis benefícios e riscos, considerando que o participante compreendeu a minha explicação.

_____ _____ _____

Data Nome do Médico Assinatura do Médico

Foi entregue um duplicado deste documento ao doente/representante legal.

ANNEX II

DEXA Informed Consent

INFORMAÇÃO AO DOENTE

(para realização de densitometria óssea)

MECANISMOS COMUNS SUBJACENTES À ATEROGÉNESE E À OSTEOPOROSE

Objectivo do Estudo

O presente estudo tem por objectivos compreender a relação entre a doença aterosclerótica e a remodelação óssea, contribuindo para uma melhoria das abordagens terapêuticas. Este estudo envolverá doentes submetidos a endarterectomia no Serviço de Cirurgia Vascular do Hospital de Santa Maria.

Descrição e Métodos do Estudo:

Os participantes neste estudo serão doentes submetidos a endarterectomia no serviço de cirurgia vascular do Hospital de Santa Maria que se voluntariem para o procedimento que a seguir se descreve.

Durante o internamento pré-operatório o participante realizará uma densitometria óssea para avaliação da densidade mineral óssea. O aparelho que determina a densidade mineral óssea produz dois feixes de raios-X de baixa intensidade e mede a quantidade destes raios que passa através do osso.

Riscos previsíveis

A densiometria óssea não apresenta efeitos secundários, já que a radiação que se utiliza actualmente não é significativa.

Possíveis Benefícios para os Participantes

Não se garante que este estudo envolva quaisquer benefícios directos para o participante. Contudo, a sua participação proporcionará informações, que poderão vir a beneficiar terceiros.

Colheita de Amostras

Os dados recolhidos durante este estudo serão utilizados para fins de investigação, não tendo o participante direitos ou benefícios financeiros que possam vir a resultar desta investigação.

Participação Voluntária e Direitos de Recusa da Participação ou de Abandono

Terá toda a liberdade para recusar a participação no estudo ou retirar o seu consentimento, suspendendo a participação em qualquer momento. A participação é

voluntária e a sua recusa em participar não envolverá qualquer penalização ou perda de benefícios. A recusa ou abandono não colocarão, de modo algum, em risco o direito a receber tratamento ou assistência médica, presentemente ou no futuro, nesta instituição.

Confidencialidade

Toda a informação sobre o participante será recolhida e analisada como parte deste ensaio. Esta informação poderá ser recolhida e combinada com informação de outros participantes. Toda a informação que identifica o participante será guardada pelo médico, juntamente com o processo clínico e será mantida confidencial.
A identificação no estudo será efectuada com as iniciais do nome e/ou com um número. Esta informação poderá ser analisada por pessoas ou entidades autorizadas pelo investigador e, nalgumas instâncias, pela Comissão de Ética deste hospital, pelas Autoridades de Saúde, sob supervisão do médico, com o objectivo de confirmar a veracidade dos dados do estudo. Toda a informação que não identifica o participante será recolhida durante o estudo e guardada numa base de dados, podendo vir a fazer parte dos resultados publicados. O nome do participante não será identificado em nenhum relatório ou publicação decorrente deste estudo. Estes dados poderão também fazer parte de projectos de investigação futuros e poderão ser comunicados a Autoridades Regulamentares.

A Quem Deve Colocar Questões Relacionadas com Este Estudo

Se tiver qualquer dúvida sobre este estudo poderá contactar: Prof. Dr.ª Helena Canhão, Telefone 217999544, Extensão 47221, Fax 217999412, e-mail: helenacanhao@gmail.com

DECLARAÇÃO DE CONSENTIMENTO INFORMADO
(para realização de densitometria óssea)

MECANISMOS COMUNS SUBJACENTES À ATEROGÉNESE E À OSTEOPOROSE

INVESTIGADOR: _____HOSPITAL: _____
NOME DO DOENTE: _____
NÚMERO DE ESTUDO DO DOENTE: _____

Eu, declaro ter tomado conhecimento e aceitar participar num estudo clínico que tem por objectivo compreender a relação entre a doença aterosclerótica e a remodelação óssea, contribuindo para uma melhoria das abordagens terapêuticas. Para esse efeito aceito que me seja feito uma densitometria óssea. O estudo proposto foi-me claramente explicado. Foi-me dada a oportunidade de colocar questões. Declaro que aceito participar, voluntariamente, neste estudo.

Recebi uma cópia desta declaração de consentimento informado, devidamente assinada e datada.

Data
Assinatura do Doente/Representante Legal

Discuti este estudo de investigação com o participante e/ou o seu representante legal, utilizando uma linguagem compreensível e apropriada. Informei adequadamente o participante sobre a natureza deste estudo e sobre os seus possíveis benefícios e riscos, considerando que o participante compreendeu a minha explicação.

Data Nome do Médico Assinatura do Médico

ANNEX III

Clinical Protocol

INQUÉRITO

ATHEROGENESIS AND OSTEOPOROSIS COMMON UNDERLYING MECHANISMS

CV_____

DADOS EPIDEMIOLÓGICOS DO DOENTE

Nome: _____

NSC: _____ **Data de Nascimento:** _____/_____/_____

Raça: _____

Sexo: ☐ **M** ☐ **F**

Profissão: _____

Escolaridade (nº anos): _____

Contacto: _____

Causa da cirurgia actual: _____

Data da cirurgia actual: _____/_____/_____ **Hora:** ____:____

Local da cirurgia: _____

Cirurgião Vascular assistente: _____

RISCO CARDIOVASCULAR

1. Idade: _____

2. PA sistólica (mmHg): _____ PA diastólica (mmHg): _____

3. Tabagismo: □ Fumador □ Não fumador

4. Colesterol Total (mg/dL): _____

5. HDL (mg/dL): _____

6. LDL (mg/dL): _____

7. Triglicéridos (mg/dL): _____

8. Glicemia em jejum (mg/dL): _____

9. PCR (mg/dL): _____

14. Creatinina (mg/dL): _____

15. Ureia: _____

16. Ácido Úrico: _____

10. Homocisteína: _____

11. Cálcio: _____

12. Vitamina D: _____

13. Coagulação:

APTT (tempo doente / tempo controlo): _____ / _____ seg.

TP (tempo doente / tempo controlo): _____ / _____ seg.

INR: _____

Altura: _____ cm Peso: _____ Kg % massa gorda: _____

IMC: _____ Perímetro abdominal: _____ cm

FRACTURAS PRÉVIAS

Já fez fractura após um traumatismo mínimo ou espontâneas (p.ex queda na rua)?

☐ Não ☐ Sim

☐ Anca ☐ Coluna ☐ Punho ☐ Úmero proximal

Data: _____/_____/_____

Idade: _____ anos

Outras fracturas prévias: ☐ Não ☐ Sim

Idade, ossos que fracturou e descrição da fractura _____

_____

_____

HISTÓRIA FAMILIAR DE OSTEOPOROSE

Algum dos seus pais fracturou o colo do fémur (anca)?

☐ Não sabe ☐ Não ☐ Sim

Qual: ☐ Pai ☐ Mãe

HÁBITOS ALIMENTARES E ESTILO DE VIDA

HÁBITOS ALCOÓLICOS

Consome ou consumiu: ☐ > 3 unidades/dia ☐ < 3 unidades/dia

Entre que idades? _____

HÁBITOS TABÁGICOS

Fuma: ☐ Não ☐ Sim Nº cigarros/dia: _____

Entre que idades? _____

Se ex-fumador: Nº cigarros/dia: _____

Entre que idades? _____

NUTRIÇÃO

Nº copos leite/dia: _____

Nº iogurtes/semana: _____

Nº fatias de queijo/semana: _____

Come regularmente vegetais? ☐ Não ☐ Sim

Come carne ou peixe todos os dias? ☐ Não ☐ Sim

Dieta: ☐ Pouco sal ☐ Sal normal ☐ Salgada

Nº cafés/dia: _____ Nº chávenas chá preto/dia: _____

ACTIVIDADE FÍSICA

Actualmente tem: ☐ Vida sedentária ☐ Vida activa

Prática regular de desporto: ☐ Não ☐ Sim

Nº horas/semana: _____ Tipo de desporto: _____

Já fez actividade física regular? ☐ Não ☐ Sim

Que actividade? _____ Nº horas/semana: _____

Entre que idades? _____

A sua profissão obriga a efectuar esforço físico intenso? ☐ Não ☐ Sim

O doente está ou esteve acamado ou imobilizado por mais de 3 meses?

☐ Não ☐ Sim Qual o motivo? _____

SE MULHER:

Idade menopausa: _____ anos ☐ Espontânea ☐ Cirúrgica

QUEDAS

Queda nos últimos 3 meses ☐ Não ☐ Sim Nº quedas: _____ Como? _____

6 meses ☐ Não ☐ Sim Nº quedas: _____ Como? _____

12 meses ☐ Não ☐ Sim Nº quedas: _____ Como? _____

ANTECEDENTES PESSOAIS

1. Artrite Reumatóide ☐ Não ☐ Sim Idade início: _____ anos

Médico Assistente: _____

2. Lúpus Eritematoso Sistémico ☐ Não ☐ Sim Idade início: _____ anos

Médico Assistente: _____

3. Outras doenças inflamatórias sistémicas crónicas ☐ Não ☐ Sim

Quais? _____ Idade de início: _____ anos

4. HTA ☐ Não ☐ Sim

5. EAM ☐ Não ☐ Sim

6. AVC ☐ Não ☐ Sim

7. Dislipidemia ☐ Não ☐ Sim Qual o tipo? _____

8. Outras doenças cardiovasculares ☐ Não ☐ Sim Qual o tipo? _____

9. Insuficiência renal ☐ Não ☐ Sim Hemodiálise? _____

10. Patologia de tiróide/paratiróide ☐ Não ☐ Sim Qual? _____

11. Outras doenças endócrinas ☐ Não ☐ Sim Qual? _____

12. Má absorção gastro-intestinal (ex: dça Celiaca, fibrose quistica) ☐ Não ☐ Sim

Qual? _____

13. Neoplasias ☐ Não ☐ Sim Qual? _____ Idade início: _____ anos

14. Doença óssea metabólica (osteoporose, doença óssea de Paget) ☐ Não ☐ Sim

Qual? _____ Idade início: _____ anos

15. Outras doenças actuais: ☐ Não ☐ Sim (especificar doença e idade de início)

_____

16. Outras doenças no passado: ☐ Não ☐ Sim (especificar doença e idade de início)

_____

17. Corticoterapia oral por mais de 3 meses: ☐ Não ☐ Sim

Dose máxima diária _____

ANTECEDENTES FAMILIARES

1. Artrite Reumatóide ☐ Não ☐ Não sabe ☐ Sim
2. Lúpus Eritematoso Sistémico ☐ Não ☐ Não sabe ☐ Sim
3. Outra doença reumática ☐ Não ☐ Não sabe ☐ Sim

Qual?

_____

5. HTA ☐ Não ☐ Não sabe ☐ Sim
6. EAM ☐ Não ☐ Não sabe ☐ Sim
7. AVC ☐ Não ☐ Não sabe ☐ Sim
8. Dislipidemia ☐ Não ☐ Não sabe ☐ Sim

Qual o tipo? _____

9. Outras doenças cardiovasculares ☐ Não ☐ Não sabe ☐ Sim

Qual? _____

10. Aneurismas ☐ Não ☐ Não sabe ☐ Sim

TERAPÊUTICA ACTUAL

_____

_____

TERAPÊUTICA

TERAPÊUTICA (POR MAIS DE 3 MESES)

	S		N	?	
Glicorticóide					Dose _____ Data Inicio _____ Duração _____ Qual? _____
Anticoagulantes					Dose _____ Data Inicio _____ Duração _____ Qual? _____
Bifosfonato					Dose _____ Data Inicio _____ Duração _____ Qual? _____
SERM					Dose _____ Data Inicio _____ Duração _____
Estrôncio					Dose _____ Data Inicio _____ Duração _____
Teriparatida					Dose _____ Data Inicio _____ Duração _____
Suplemento Ca					Dose _____ Data Inicio _____ Duração _____
Vitamina D					Dose _____ Data Inicio _____ Duração _____
B-bloqueantes					Dose _____ Data Inicio _____ Duração _____ Qual? _____
IECA					Dose _____ Data Inicio _____ Duração _____ Qual? _____
ARA					Dose _____ Data Inicio _____ Duração _____ Qual? _____
Diuréticos					Dose _____ Data Inicio _____ Duração _____ Qual? _____
Vasodilatadores					Dose _____ Data Inicio _____ Duração _____ Qual? _____
Hipolipidemiantes					Dose _____ Data Inicio _____ Duração _____ Qual? _____
Estatinas					Dose _____ Data Inicio _____ Duração _____
AAS					Dose _____ Data Inicio _____ Duração _____
Antidiabéticos orais/insulina					Dose _____ Data Inicio _____ Duração _____
Denosumab					Dose _____ Data Inicio _____ Duração _____

Outras terapêuticas actuais ou anteriores (especificar terapêutica e data de início): _____

Data de preenchimento do protocolo: _____/_____/_____ Assinatura: _____

ANNEX IV

Alizarin Red S Staining Protocol for Calcium

Alizarin Red S Staining – Protocol [49]

Description
Alizarin Red Solution is used for histological calcium identification. Magnesium, manganese, barium, strontium and iron are chemical elements which might interfere with the reaction, besides their insufficient concentration.

Fixation	Positive Control
Neutral or alcoholic formalin. Paraffin.	Mouse embryo or other known structure containing calcium.

Solution and Reagents
Alizarin Red Solution Alizarin Red S (C.I. 58005) – 2 g Distilled water – 100 mL Mix the Alizarin Red S with the distilled water. Then, verify the pH, and adjust it to 4,1 – 4,3, if necessary, with 10% ammonium hydroxide.
Acetone (100%)
Acetone-Xylene Acetone (100%) – 50 mL Xylene – 50 mL

Procedure
1. Slowly deep the slides in distilled water; 2. With the Alizarin Red Solution stain the slides for 2 minutes (30 seconds to 5 minutes). Then, use the microscope to observe the reaction; 3. Get rid of excess colorant and blot sections; 4. First, dehydrate in acetone – 20 dips. Then, deep 20 times in Acetone-Xylene solution; 5. Clear in xylene for 10 minutes. Afterwards, mount using *Entellum*® (or other synthetic mounting medium).

Results
Calcium deposits produce orange-red stain. The precipitate is birefringent.